Design and development of a simple laboratory model to detect ^{15}N enrichment in cyanobacterial biomass and extra cellular ammonia using ^{15}N gas

G. Selvakumar*§ · G. Gopalaswamy · K. Arulmozhiselvan

Received: 17 July 2006 / Final revision: 10 December 2006 / Accepted 19 December 2006

Abstract A laboratory scale working model that could detect the ^{15}N enrichment in cyanobacterial biomass and extracellular ammonia, using ^{15}N gas under *in vitro* conditions was designed and fabricated. Using the model, ^{15}N enrichment of 0.48% atom excess was detected in the cyanobacterial biomass on the 30 d after inoculation. The ^{15}N enrichment increased linearly in the extracellular ammoniacal fraction from the 20 d onward. The model would prove to be a useful tool to quantify the extent of ^{15}N enrichment under *in vitro* conditions using ^{15}N gas.

Key words · Cyanobacteria · laboratory model · ^{15}N enrichment · ^{15}N gas

Introduction

Cyanobacteria commonly referred as Blue Green Algae due to their ability to form phycobilin pigments are a group of photosynthetic prokaryotes. They have colonized the surface of the earth for nearly 3 billion years and are considered the predecessors of modern day cellular organelles¹. Taxonomically they belong to the class cyanophyceae².

A unique property of certain cyanobacterial genera is their nitrogen fixing ability. This is closely related to the presence of specialized cells called heterocysts, which lack PS II and hence do not evolve oxygen. It is in these cells that nitrogenase, a highly oxygen sensitive enzyme is protected. Though all heterocystous cyanobacteria can fix atmospheric nitrogen under aerobic conditions, a few non-heterocystous filamentous and unicellular cyanobacteria also possess this property³. Cyanobacteria gain agronomic significance due to their ability to colonize and contribute to the biological nitrogen fixation in rice fields. The biologically fixed elemental nitrogen is made available to the rice crop either after the mineralization of the cyanobacterial biomass or continuously as extracellular ammonia. Hence it is imperative to quantify the cellular and extra cellular fractions of the biologically fixed nitrogen.

Biological nitrogen fixation can be estimated by either direct or indirect methods⁴. The Acetylene Reduction Assay (ARA) is an indirect method of quantifying biologically fixed nitrogen. But this method has several inherent flaws and the results may be quite erroneous⁵. The use of isotope techniques make it possible to quickly and accurately find out the answer to soil-crop-fertilizer management problems, which cannot be solved by other conventional techniques⁶. The ^{15}N technique has been successfully applied to a number of tropical and temperate crop and forage legumes and N_2 fixing trees both in agro-forestry systems

G. Selvakumar*§ (✉)

*Department of Agricultural Microbiology,
Tamil Nadu Agricultural University,
Coimbatore - 641 003

e-mail: gselva74@rediffmail.com,

Tel: +91 / 5962 / 230060

Present Address: §VPKAS (ICAR), Almora - 263 601

G. Gopalaswamy · K. Arulmozhiselvan

#Agricultural College and Research Institute,
Madurai - 625 004

and natural ecosystems⁷. The technique has been demonstrated to provide accurate estimates of nitrogen fixation as other methodologies⁸.

^{15}N gas has been used previously to estimate nitrogen fixation associated with deep-water rice to determine the distribution of nitrogen compound produced by the cyanobacterial symbiont of *Azolla*⁹. The technique has been used to quantify the amount of biological nitrogen fixation in rice production systems in a fairly accurate manner¹⁰. In this study, a laboratory model was designed and developed to detect the ^{15}N enrichment in cyanobacterial biomass and extra cellular ammonia, using ^{15}N gas (Fig. 1). In the laboratory model, the ^{15}N gas level was raised above the natural abundance (0.3665 %), by evolving ^{15}N gas for a period of 30 d at daily intervals. At the end of the 30 d period the ^{15}N was estimated in the cellular and extra cellular fractions of the cyanobacteria. The ^{15}N enrichment in the extracellular ammoniacal fraction was determined at 10 d intervals.

Materials and Methods

Design of the bioreactor

The bioreactor consisted of four distinct parts viz., (A) ^{15}N gas generation unit (B) Bromine Absorption Unit (C)

Cyanobacterial Growth Chamber and (D) Volatilizable Ammonia Receiving Unit (Fig.1.).

^{15}N gas generation unit

A specially designed 25 mL conical flask with a ground joint was used to generate ^{15}N gas. Sodium hypobromite (10 mL) was taken in the flask and 200 mg ^{15}N urea (10 % atom excess obtained from Rashtriya Chemicals and Fertilizers) dissolved in 5 mL distilled water was injected through a rubber septum to generate ^{15}N gas daily for a period of 30 d. The rubber septum was periodically replaced to ensure airtight conditions.

Bromine Absorption Unit

In order to absorb any trace quantity of bromine that may have been present as a contaminant in the ^{15}N gas generated, it was passed through the bromine absorption unit, which contained 0.1 N HCl (10 mL). The acid was replaced at weekly intervals.

Cyanobacterial Growth Chamber

A three-litre Haffkine flask was used as the growth chamber. Sterile BG-11₀ medium (1.5 L) prepared by using double

Fig. 1 Laboratory model to detect ^{15}N enrichment in cyanobacterial biomass and extracellular ammonia using ^{15}N gas.

- (A)- ^{15}N gas generation unit (25 mL)
- (B)- Bromine absorption unit (25 mL)
- (C)-Cyanobacterial growth chamber (3 L)
- (D)-Volatilizable ammonia collection unit (250 mL)

(a) Ground joint; (b) Injectible septum; (c) Pinch cork; (d) Sampling port; and (e) Nylon septum.

distilled water, was taken in the flask and inoculated with a composite cyanobacterial inoculum at 10% level. The inoculum contained members of the genus *Westiellopsis* that were tolerant to various stresses like acidity, herbicide and salinity. The flask was fitted with a three holed nylon stopper and the entire assembly was made airtight with the application of vacuum crease.

Volatilizable Ammonia Receiving Unit

A specially designed conical flask (250 mL) with a ground joint served as the volatilizable ammonia-receiving unit. Boric acid (2%) with double indicator (bromocresol green 0.5 g and methyl red 0.1 g in 100 mL of ethyl alcohol) was kept at the receiving end to absorb the ammonia that volatilized from the growth chamber.

Estimation of ^{15}N enrichment in cyanobacterial growth medium and cyanobacterial biomass

The ammoniacal nitrogen content in the growth medium was estimated at 10 d interval for a period of 30 d by the distillation method¹¹. The cyanobacterial biomass was harvested on the 30th d by filtration and the total N content in the cyanobacterial biomass was estimated by the method of Humpries¹². On the 30 d, the contents of the volatilizable ammonia receiver were transferred to a Bremners distillation assembly and the ammoniacal nitrogen content was estimated by the distillation method¹¹. The acidified distillate obtained from each of the fractions was dried separately in a sand bath at 60°C to get the ammonium borate crystals, which were stored in polybags for $^{15}\text{N}/^{14}\text{N}$ ratio analysis. The ammonium borate crystals were reacted with sodium hypobromite in a mass spectrometer (Micromass 622 VG ISOGAS) to quantify the $^{15}\text{N}/^{14}\text{N}$ ratio. The nitrogen gas evolved by the reaction was fed into the inlet of the mass spectrometer under vacuum condition. The

current of the major ion beam (M) and minor ion beam (m) were recorded and ^{15}N abundance was calculated using the formula.

$$R = \frac{m}{(M+1) \times 100}$$

$$^{15}\text{N} \text{ atom excess} = \frac{R}{R + 2}$$

From the ^{15}N atom excess the labeled N recovered by the cyanobacteria was calculated as per standard procedures¹³. The ^{15}N % atom excess in each of the individual components was worked out by detecting the observed ^{15}N enrichment from the natural enrichment (0.3663%). The results were expressed as % atom excess.

Results and Discussion

It was observed that the nitrogen content of the composite cyanobacterial cell biomass that was labeled using ^{15}N gas had a nitrogen content of 5.64 % and the ^{15}N percent atom excess in the labeled biomass was 0.48 % (Table 1). This indicates the efficiency of labeling the cyanobacterial biomass by ^{15}N gas and this is a direct measure of the quantity of the ^{15}N gas incorporated into the cell biomass through cyanobacterial nitrogen fixation. It would be interesting to note that the cyanobacteria were grown in a N free medium and the observed nitrogen content and the ^{15}N % atom excess could be solely attributed to nitrogen fixed by the cyanobacteria. The ammoniacal nitrogen content of the medium increased linearly with the incubation period. Maximum ammoniacal nitrogen content in the medium was observed on the 30th d after inoculation. Interestingly, no ^{15}N could be detected on the 10 d in the ammoniacal fraction, while a steady increase was observed in the ^{15}N percent atom excess in the ammoniacal fraction on the 20 and 30 d after inoculation. The ammonia that volatilized from the growth chamber was collected in 2 % boric acid solution. At the end of the incubation pe-

Table 1 Nitrogen content and ^{15}N per cent atom excess in the labeled cyanobacterial cell biomass and ammoniacal fractions in the laboratory model.

Biomass fresh weight (g)	Total N (%)	% Atom excess in biomass	Ammoniacal N content in media (ppm)			¹⁵ N % atom excess in ammoniacal N			Volatalized NH ₃ content (ppm)	% Atom excess in volatalized NH ₃
			Day interval							
At the end of 30 d incubation			10	20	30	10	20	30	At the end of 30 d incubation	
2.0	5.64	0.48	5.6	11.2	14.7	BDL	0.11	0.26	16.8	0.30

BDL- Below Detectable Level.

riod (30 d) it was observed that the ammoniacal nitrogen content was 16.8 ppm. The ^{15}N atom excess in this fraction collected during the entire incubation period was 0.30 %.

The use of ^{15}N gas in growth chambers has been advocated for the short-term incubation studies under controlled conditions to study the nitrogen fixation by microbes and to work out the nitrogen derived by plants by biological nitrogen fixation¹⁴. Few studies have been conducted with cyanobacteria using ^{15}N gas. Earlier studies with preparations of the isolated cyanobacterial symbiont *Anabaena azollae* have shown that only 50 % of the fixed ^{15}N is incorporated into an organic fraction while about 35 % is released into the medium as ammonium¹⁵. *Anabaena* preparations isolated from *Azolla caroliniana* incubated for 10 min. with $^{13}\text{N}_2$ gas resulted in the formation of labeled radio active compounds viz., ammonium, glutamine, glutamate and alanine, of which ammonium accounted for 66 % of the total labeled compound and 58 % of which was in the extra cellular fraction¹⁶. In the present study a novel laboratory model was designed and developed to detect the ^{15}N enrichment in the cyanobacterial biomass and the extracellular ammonia fraction. In this model boric acid was used to collect the volatilized ammoniacal fraction that evolved over a period of 30 d thus reducing the possibility of underestimating the ammonia production. It was observed that the cyanobacterial biomass had a ^{15}N enrichment of 0.48%, which would have come entirely through biological nitrogen fixation. Interestingly, the ^{15}N enrichment of 0.11 and 0.26 % was also detected in the extracellular ammonia on the 20 and 30 d after incubation and 0.30 % of the ^{15}N enrichment was detected in the volatilized ammoniacal fraction, which was estimated on the 30 d. The findings of the present study are in conformity with the earlier observations that ^{15}N enrichment could be detected in the extracellular fraction and the cell biomass, when ^{15}N gas was used as a tracer. The model that was designed and developed during the present study will serve as a tool for assessing the extent of nitrogen fixed in the cyanobacterial biomass /excreted in the extracellular ammoniacal fraction under laboratory conditions.

Acknowledgements The authors wish to thank Dr. P. Balasubramanian, Director, Centre for Plant Molecular Biology, TNAU for his kind help and support during the study.

References

1. Haselkorn R (1978) Heterocysts. Annu. Rev. Plant Physiol 29:319–344.
2. Desikachary TV (1959) Cyanophyta, Indian Council of Agric. Res, New Delhi, India.
3. Anand N (1997) Nitrogen fixation by non-heterocystous cyanobacteria. In: Advances in Phycology, (Verma, BN, Kargupta AN & SK Goyal eds). APC Publication, New Delhi, India, pp 187–197.
4. Danso AKS (1995) Assessment of biological nitrogen fixation. Fert Res 42:33–41.
5. Giller KE & Wilson KI (1991) Nitrogen fixation in tropical cropping systems. CAB International, Oxon, U.K, 1 p.
6. Sinha MN (2001) Isotope ^{15}N for agronomic research: An overview. J Nuclear Agric Biol 30: 121–142.
7. Ladha JK, Peoples MB, Garrity DP, Capuno VT & Dart PJ (1993) Estimating dinitrogen fixation of hedgerow vegetation in an alley-crop system using the ^{15}N natural abundance method. Soil Sci Soc Am J 57:732–737.
8. Doughton JA, Saffigna PG, Vallis I & Mayer R J (1995) Nitrogen fixation in chickpea II. Comparisons of ^{15}N enrichment and ^{15}N natural abundance method for estimating nitrogen fixation. Aust J Agri Res 46:225–236.
9. Watanabe I, Baikizhi, Berja WS, Espinas CR, Ito O & Sabudhi BPR (1982) Recent studies in *Azolla-Anabaena* complex in rice culture. IRRI Res Paper Series 69.
10. Kamuru F, Albrecht SL, Baker JT, Allen LH & Shanmugam KT (1994) Growth responses of paddy rice to an ammonia-excreting mutant cyanobacterium at elevated CO_2 concentration. Applied Soil Ecol 1:199–206.
11. Bremner JM & Keeney DR (1982) Determination and isotope - ratio analysis of different forms of nitrogen in soils. Exchangeable ammonium nitrate and nitrite by extraction distillation method. Soil Sci Soc Amer Proc 30:577–582.
12. Humphries EC (1956) Mineral components and ash analysis. In Modern methods of plant analysis, Vol I, (K Peach & MV Tracey eds). Springer Verlag, Berlin, Germany, pp 468–502.
13. Pruden G, Powlson DS & Jenkinson DS (1985) The measurement of ^{15}N in soil and plant material. Fert Res 6:205–218.
14. Arulmozhiselvan K (2004) Estimation of biological nitrogen fixation by ^{15}N isotope technique. In Biofertilizers technology for rice based cropping systems, (S Kannaiyan, K Kumar & K Govindarajan eds). Scientific Publishers, Jodhpur, India, pp 169–181.
15. Peters GA, Ray TB, Mayne BC & RE Toia Jr (1980) *Azolla - Anabaena* association: Morphological and Physiological studies. In Nitrogen fixation Vol. 2, (WE Newton & WH O Johnson eds). University Park Press, Baltimore, USA, pp 293–309.
16. Meeks JC, Wolk CP, Thomas J, Lockaun W, Lockaun PW, Shaffer PW, Austin SM, Chien WS & Galonsky A (1977) The pathways of assimilation of NH_4^+ by the cyanobacterium *Anabaena cylindrica*. J Biol Chem 252:7894–7900.